RESEARCH ARTICLE

The early humoral immune response to *Bacillus anthracis* toxins in patients infected with cutaneous anthrax

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Abstract

Bacillus anthracis, the causative agent of anthrax, produces a tripartite toxin composed of two enzymatically active subunits, lethal factor (LF) and edema factor (EF), which, when associated with a cell-binding component, protective antigen (PA), form lethal toxin and edema toxin, respectively. In this preliminary study, we characterized the toxin-specific antibody responses observed in 17 individuals infected with cutaneous anthrax. The majority of the toxin-specific antibody responses observed following infection were directed against LF, with immunoglobulin G (IgG) detected as early as 4 days after the onset of symptoms in contrast to the later and lower EF- and PA-specific IgG responses. Unlike the case with infection, the predominant toxin-specific antibody response of those immunized with the US anthrax vaccine absorbed and UK anthrax vaccine precipitated licensed anthrax vaccines was directed against PA. We observed that the LF-specific human antibodies were, like anti-PA antibodies, able to neutralize toxin activity, suggesting the possibility that they may contribute to protection. We conclude that an antibody response to LF might be a more sensitive diagnostic marker of anthrax than to PA. The ability of human LF-specific antibodies to neutralize toxin activity supports the possible inclusion of LF in future anthrax vaccines.

Introduction

Anthrax is a zoonotic disease caused by *Bacillus anthracis*, a Gram-positive spore-forming microorganism whose manifestations in humans depend on the route of infection. The cutaneous form of the disease accounts for more than 95% of reported cases (Shafazand *et al.*, 1999) and, with treatment, does not usually pose a threat to human life (Little & Ivins, 1999). The gastrointestinal and inhalational forms of the disease, although not as common, are much more severe (Little & Ivins, 1999). The ability of the organism to form environmentally resistant spores, be dispersed as aerosols and cause lethal infection following inhalation has resulted in its development and use as a biological weapon.

Following infection, spores are phagocytosed by macrophages and transported to the draining lymph nodes, where they germinate into vegetative bacilli and escape from the macrophage (Lincoln *et al.*, 1965; Dixon *et al.*, 2000; Guidi-Rontani *et al.*, 2001). In cutaneous anthrax, this results in a localized infection; in inhalational anthrax, the bacilli multiply in the lymphatic system and spread to the blood, resulting in massive bacteremia and toxemia (Fish & Lincoln, 1968).

Within 3 h of spore germination, the expression of the toxin proteins begins (Guidi-Rontani *et al.*, 1999). The extracellular tripartite toxin of anthrax is composed of two enzymatically active subunits, lethal factor (LF) and edema factor (EF), and a cell-binding and translocation component, protective antigen (PA). Both lethal (PA+LF) and edema (PA+EF) toxins are able to suppress key parts of the innate immune response to the developing infection (O'Brien *et al.*, 1985; Wright & Mandell, 1986; Duesbery

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14. ABSTRACT

Bacillus anthracis, the causative agent of anthrax, produces a tripartite toxin composed of two enzymatically active subunits, lethal factor (LF) and edema factor (EF), which, when associated with a cell-binding component, protective antigen (PA), form lethal toxin and edema toxin, respectively. In this preliminary study, we characterized the toxin-specific antibody responses observed in 17 individuals infected with cutaneous anthrax. The majority of the toxin-specific antibody responses observed following infection were directed against LF, with immunoglobulin G (lgG) detected as early as 4 days after the onset of symptoms in contrast to the later and lower EF- and FA-specific IgG responses. Unlike the case with infection, the predominant toxin-specific antibody response of those immunized with the US anthrax vaccine absorbed and UK anthrax vaccine precipitated licensed anthrax vaccines was directed against PA. We observed that the LF-specific human antibodies were, like anti-PA antibodies, able to neutralize toxin activity, suggesting the possibility that they may contribute to protection. We conclude that an antibody response to LF might be a more sensitive diagnostic marker of anthrax than to PA. The ability of human LF-spedfic antibodies to neutralize toxin activity supports the possible inclusion of LF in future anthrax vaccines.

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et al., 1998; Pellizzari *et al.*, 1999; Erwin *et al.*, 2001; Kalns *et al.*, 2002; Popov *et al.*, 2002; Moayeri *et al.*, 2003). Later in the disease process, high levels of lethal toxin (LT) induce the cytokine independent shock-like death associated with anthrax (Moayeri *et al.*, 2003).

Animal studies suggest that as the concentration of toxin increases the likelihood of successfully treating an infected individual decreases until it reaches a level at which antibiotics are no longer effective (Albrecht *et al.*, 2007; Baillie, 2009). Given that the early detection of toxin is a key diagnostic marker, it surprising how little is known of the time course of toxin production in humans and thus we sought to characterize the early immune responses to individuals infected with anthrax (Baillie, 2009).

To achieve this aim, clinical serum samples previously obtained at various time points postinfection from individuals who had contracted cutaneous anthrax were examined for the presence of toxin-specific immunoglobulin M (IgM) and IgG antibodies. In addition, we compared these antibody responses with those seen following immunization with the US anthrax vaccine absorbed (AVA) and UK anthrax vaccine precipitated (AVP) licensed human anthrax vaccines. Finally, the protective function of the toxin-specific antibody responses stimulated following infection were assessed using an assay that measures toxin neutralization, a recently demonstrated correlate of protection (Reuveny *et al.*, 2001; Little *et al.*, 2004).

Materials and methods

Expression and purification of toxin components

The PA, LF and EF genes were cloned into the *Escherichia coli* expression vector pQE-30 (Qiagen) and confirmed by sequencing (Read *et al.*, 2003). Proteins were expressed from either the M15 (PA) or the SG13009 (LF and EF) strain of *E. coli*. Host strains were grown in Luria–Bertani medium to an $OD_{600\,nm}$ of 0.55–0.65 and induced with 1 mM isopropyl-β-D-thiogalactoside either 4 h at 37 °C (PA) or 20 h at 25 °C (LF and EF). Cells were pelleted and lysed by French press at 16 000 p.s.i.; lysates were cleared at 45 000 *g* for 15 min. Recombinant proteins were purified by cobalt affinity chromatography. Cleared lysate was batch bound to TALON resin (Clontech) and then washed with 10 CV 300 mM NaCl, 50 mM Na_2HPO_4 and 20 mM imidazole, pH 7.0. Proteins were eluted in 5 CV 300 mM NaCl, 50 mM Na_2HPO_4 and 150 mM imidazole, pH 7.0. Fractions containing protein [determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] were pooled and dialyzed into 10 mM HEPES and 50 mM NaCl, pH 7.5. Proteins purified using this procedure were approximately 90% pure as assessed by SDS-PAGE with Coomassie staining.

Serum samples

Serum samples were obtained from volunteers who had received at least a priming series of the AVA (six Maryland-based volunteers) or AVP (four UK-based volunteers visiting Maryland) vaccines. Control samples were obtained from six nonimmunized, noninfected Maryland-based individuals. All samples were obtained under a protocol approved by the University of Maryland and the Naval Medical Research Center's Institutional Review Boards, as well as by the Ethics Committee at Erciyes University. Informed consent was obtained from all individuals. Clinical samples were obtained from 17 cutaneous anthrax patients attending the infectious diseases clinic at Erciyes University in Turkey (Table 1). Serum samples were not collected prospectively from patients under a set protocol, but were instead collected when patients presented to the outpatient clinics for up to 21 days after the initial visit. Anthrax was diagnosed by exposure history, clinical presentation consistent with anthrax, Gram stain and positive culture from the lesion.

Antitoxin IgG and IgM enzyme-linked immunosorbent assay (ELISA)

Antitoxin IgG and IgM levels were measured using an ELISA as described previously, with minor variations (Hepburn *et al.*, 2007). Data values were compared with a standard curve of purified human IgG or IgM (Sigma). Data in the linear portion of the ELISA graph and within the range of the standard curve were used to calculate the quantitative titer ($\mu g\,mL^{-1}$) for the serum sample. For each antigen, four to six naïve serum samples were assayed and their titers were averaged (geometric mean) and the 95% confidence interval of the distribution was calculated. Experimental data were scored as a positive result only if the calculated titer exceeded the upper limit of the confidence interval of the naïve control samples.

LT neutralization assay

The toxin neutralization assay was performed on the mouse monocyte cell line J774A.1 (ATCC) as described previously, with cell viability determined by the addition of DMEM containing sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (Roche) for 16 h. The assay was read at 480 nm. The dilution series data ($A_{480\,nm}$ vs. toxin concentration) were modeled with four-parameter logistic curves of the form:

$$OD_{480\,nm}(y) = \beta_1 + (\beta_2 - \beta_1)/[1 + \exp\{\beta_3(\beta_4 - x)\}]$$

The data were fit using MATLAB software via a nonlinear least-squares analysis, yielding the parameters (β_1, β_2, β_3,

Table 1. Details for the patients with cutaneous anthrax

Patient no.	Incubation period (days)	*Contact with contaminated animal materials	Site of the lesion	†Gram stain for *B. anthracis*	†Positive culture for *B. anthracis*	Previous antibiotic use	‡Antibiotic initiation day of disease	Given antibiotic	Duration of therapy (days)	Outcome
1	5	+	Right eye lids	–	–	Yes	Third	Pen G	15	Left deep tissue scar, recovered
2	15	+	Right arm	–	–	Yes	Fourth	Pen G+Cipro	10	Recovered
3	1	+	Right arm	–	–	Yes	Second	Pan G	10	Recovered
4	6	+	Right hand	–	–	Yes	Fourth	Pen G	10	Recovered
5	9	+	Left hand	–	–	No	Sixth	Pen G	7	Recovered
6	10	+	Left forearm	–	–	Yes	Fifth	Pen G	10	Recovered
7	5	+	Right hand fingers	+	+	No	Fifth	Pen G	7	Recovered
8	4	+	Left hand finger	+	–	Yes	Fourth	Pen G	15	Recovered
9	12	+	Both hands	–	–	Yes	Third	Pen G	14	Recovered
10§	2	+	Anterior neck	+	+	No	Second	Pen G	14	Left deep tissue scar, recovered
11	8	+	The eye lid	+	+	No	Third	Pen G	10	Recovered
12	8	+	Left eye lids	+	–	Yes	Third	Pen G	14	Recovered
13	5	+	Right wrist	+	–	Yes	Fifth	Pen G	10	Recovered
14	?	+	Left elbow	–	–	Yes	Tenth	Pen G	10	Recovered
15	5	+	Right face	+	–	Yes	Fifth	Pen G	10	Recovered
16	1	+	Anterior neck	+	–	No	Seventh	Pen G	7	Recovered
17	7	+	Right arm and left wrist	–	–	Yes	Fourth	Pen G	10	Left deep tissue scar, recovered

*The patient gave a history of contact with an ill animal or contaminated animal materials (such as slaughtering of ill animals, skinning, chopping meat, carrying raw skin or splashing blood from dying animal during slaughtering). One case gave a history of fly bite.
†The swabs for Gram stain and culture were taken from the vesicles of fluid or under the crust if developed.
‡Time from first symptoms to the diagnosis and initiation of antibiotic treatment.
§In this case, anthrax sepsis was developed from the cutaneous lesion.
Pen G, penicillin G; Cipro, ciprofloxacin.

β_4) of the best fit. The inflection point of the fit (β_4 parameter) corresponds to the serum dilution that ensures the survival of 50% of the cells in the assay (ED_{50}) (Quinn *et al.*, 2004).

Results

Clinical characteristics

Cutaneous anthrax in the patients was diagnosed by the history of contact with ill animals and animal products, typical cutaneous lesions and demonstration of Gram-positive bacilli (eight cases) and/or -positive cultures (three cases) from the lesion (Table 1). The upper body was the usual site of the cutaneous anthrax lesion. The finding that cultures were positive only in three cases was presumed to be due to the fact that many patients had taken an antibiotic before their initial visit. With cultures of both the lesion and the blood being positive, one patient was diagnosed with anthrax sepsis originating from the cutaneous lesion. The incubation period varied between 1 and 15 days. Seventeen patients gave a history of contact with an ill animal or contaminated materials of animals that died. The time from first symptom to the initiation of antibiotics ranged from 2 to 10 days, with 16 subjects receiving antibiotics, predominantly penicillin, within the first 7 days, while 10 patients received antibiotics within 4 days. Typically, the patients were treated for 7–15 days, and all recovered. Three subjects (patients 1, 10 and 17), however, were left with deep tissue scars.

Antitoxin IgM responses

In order to examine the early immune response to infection, the serum IgM levels against PA, LF and EF were measured and compared with the antitoxin IgM titer of six naïve volunteers. Of the 17 infected individuals, six (35%) had an IgM response to at least one of the toxin components that was higher than that of the naïve controls (Table 2). There was no statistical difference in the level of toxin-specific IgM between the remaining 11 patients and the naïve controls. An anti-LF-specific IgM was detected in five individuals within the first and second weeks following symptoms, with the earliest response detected at day 4. In contrast, an anti-PA IgM was detected in four individuals, but not until the second and third weeks following symptoms, with the earliest titers appearing at day 12. Finally, anti-EF titers were detected in only two patients with titers occurring in the second week after symptoms.

Antitoxin IgG responses

The serum IgG titers against the anthrax toxin proteins in 17 infected individuals were measured and compared with the geometric mean IgG titer (GMT) of six naïve volunteers. In contrast to the IgM responses, 11 of the 17 patients (65%) demonstrated a measurable IgG titer to at least one of the toxin components on the days assayed (Table 3). While each positive patient generated an anti-LF IgG response with titers ranging from 13.6 to 817 $\mu g\,mL^{-1}$, only four individuals showed an anti-EF IgG response (patients 5, 6, 8 and 16) and only three patients (patients 2, 6 and 7) had IgG specific for PA. The anti-LF IgG response in all infected individuals exceeded that directed against PA and EF. During the second week of infection, the GMT of all responders to LF (69.3 $\mu g\,mL^{-1}$) was almost twice the anti-EF titer (37.4 $\mu g\,mL^{-1}$) and three times the anti-PA titer (22.6 $\mu g\,mL^{-1}$).

LF-specific IgG responses were detected as early as 4 days after onset of symptoms while anti-EF IgG responses were not seen until day 6 and anti-PA IgG responses first being detected at day 13. These preliminary results would appear to indicate that of the three toxins components, the antibody response to LF represents the most appropriate early diagnostic indicator.

To further characterize the protective nature of the antibody response induced by infection, we compared the spectrum of the resulting toxin-specific IgG responses to those seen following immunization of healthy volunteers with the US AVA and the UK AVP licensed human anthrax vaccines (Pittman *et al.*, 2002; Baillie *et al.*, 2003, 2004). As can be seen from Fig. 1a and b, all of the vaccinees mounted a PA-specific IgG response, the level of which varied depending on the vaccine used and the immunization schedule of each individual. In contrast to the responses observed following infection, little if any LF-specific IgG was

Table 2. Comparison of serum antitoxin IgM levels in cutaneous anthrax patients

Patient	Days after onset of symptoms	Anti-PA IgM	Anti-LF IgM	Anti-EF IgM
Naïve	–	1.2 (1.3)	4.7 (3.3)	2.2 (2.0)
1	5	< NB	< NB	< NB
	12	7.1 (1.8)	11.3 (1.1)	< NB
2	4	< NB	8.9 (0.2)	< NB
	13	2.7 (0.1)	10.2 (1.0)	4.7 (2.5)
5	6	< NB	< NB	< NB
	13	< NB	9.3 (2.0)	< NB
7	5	< NB	< NB	< NB
	15	3.4 (0.3)	8.6 (0.8)	< NB
10*	7	< NB	8.3 (0.1)	< NB
16	14	< NB	< NB	5.1 (2.3)
	21	2.6 (0.8)	< NB	< NB

Data are in $\mu g\,mL^{-1}$ and represent the mean titer ($\pm$ SD) as determined by ELISA.

*Patient developed septicemia from cutaneous lesion.

< NB, less than the naïve baseline (titer indistinguishable from naïve controls).

Table 3. Serum antitoxin IgG levels in cutaneous anthrax patients

Patient	Days after onset of symptoms	Anti-PA IgG	Anti-LF IgG	Anti-EF IgG
Naive	–	11.6 (5.4)	5.6 (4.1)	6.5 (6.0)
1	5	< NB	< NB	< NB
	12	< NB	147.4 (19.0)	< NB
2	4	< NB	< NB	< NB
	13	21.6 (6.4)	117.3 (26.8)	< NB
3	4	< NB	13.6 (3.2)	< NB
	9	< NB	16.0 (3.7)	< NB
5	6	< NB	15.7 (2.1)	14.7 (1.5)
	13	< NB	141.0 (19.3)	27.1 (2.7)
6	5	< NB	< NB	< NB
	20	26.1 (2.2)	430.8 (91.0)	24.3 (1.6)
7	5	< NB	< NB	< NB
	15	19.3 (4.8)	71.9 (18.1)	< NB
8	4	< NB	< NB	< NB
	9	< NB	35.6 (13.6)	60.5 (7.9)
	13	< NB	68.7 (18.5)	31.9 (8.2)
9	15	< NB	28.9 (9.6)	< NB
10*	7	< NB	31.0 (8.2)	< NB
16	14	< NB	214.3 (24.4)	< NB
	21	< NB	817.6 (202.8)	26.2 (7.6)
17	7	< NB	< NB	< NB
	15	< NB	60.2 (10.9)	< NB

Data are in μg mL^{-1} and represent the mean titer (± SD) as determined by ELISA.

*Patient developed septicemia from cutaneous lesion.

< NB, less than the naïve baseline (titer indistinguishable from naïve controls).

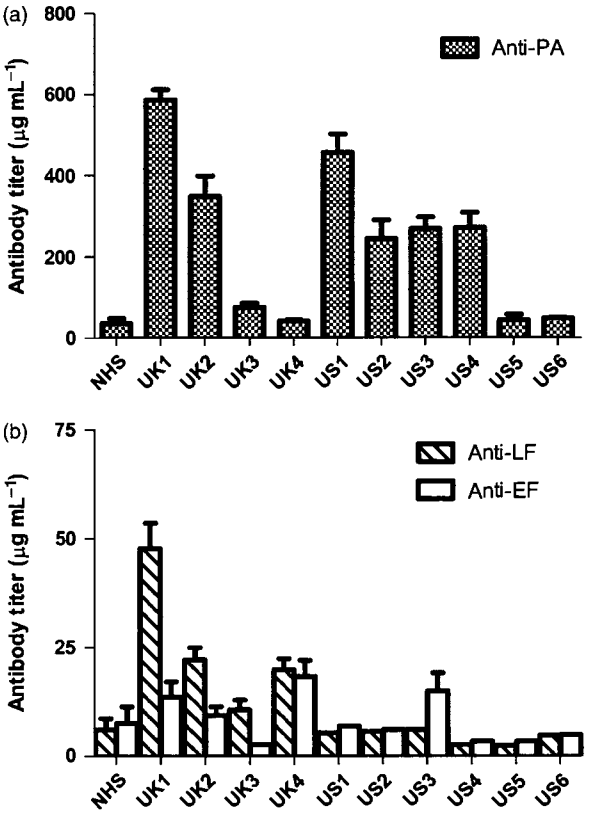

Fig. 1. Serum antitoxin titers of immunized individuals. The antitoxin responses of individuals immunized with the US AVA or the UK AVP licensed vaccine were compared. Responses to PA and LF and EF are shown in (a) and (b), respectively. Data represent the mean titer (± SD) of four trials as determined by ELISA.

detected and then only in recipients of the UK vaccine. While we were unable to draw any conclusions as to the relative efficacy of each vaccine due to the small sample size, we could conclude that PA comprised the major immunogen within the vaccine, particularly those immunized with the AVA vaccine (Fig. 1a).

Neutralization of LT

The ability of the AVA and AVP vaccines to stimulate a protective immune response has been demonstrated across a number of animal studies and is thought to be due to the production of PA-specific antibodies capable of neutralizing anthrax toxin activity (Baillie, 2009). Indeed, toxin-neutralizing PA-specific IgG antibodies have been identified as a correlate of protection (Reuveny *et al.*, 2001; Little *et al.*, 2004).

To determine whether serum from infected individuals also contained toxin-neutralizing antibodies, we assayed the activity of samples collected from 10 infected individuals at the time point that had the highest antitoxin IgG titer (Fig. 2). Neutralizing activity was detected in all samples, with ED_{50} values ranging from 103.8 (patient #8) to 7983 (patient #5). The presence of LF-specific antibodies suggested a possible correlation between LF antibody titer and toxin neutralization. However, linear regression analysis of the ED_{50} values vs. anti-LF IgG produced only a weakly positive correlation ($R^2 = 0.323$) possibly due to the small number of samples included in the analysis (Fig. 3). No correlate was observed between the ED_{50} values and anti-PA IgG, anti-PA IgM or anti-LF IgM titers (data not shown).

Discussion

Anthrax is primarily a disease of animals that occasionally infects humans and as a consequence opportunities to study the pathology of the disease in humans are rare (Baillie, 2009). The early antibody responses of a small number of individuals suffering from cutaneous anthrax were analyzed for their ability to recognize the individual components of the tripartite toxin produced by *B. anthracis*. In the context of infection, one would expect the initial antibody response to comprise IgM class antibodies and while this was true for a small number of individuals, the majority showed a mixed

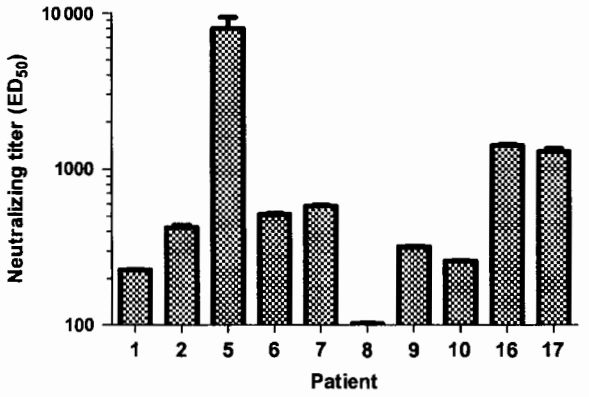

Fig. 2. Lethal toxin-neutralizing antibody titers of cutaneous anthrax patients. For patients with more than one serum sample, neutralizing titers were measured at the time point with the highest IgG titer. Neutralizing titer ED_{50} values ranged from 103.8 to 7983. Data represent the ED_{50} (β_4 parameter) generated by a four-parameter logistic fit of four tests.

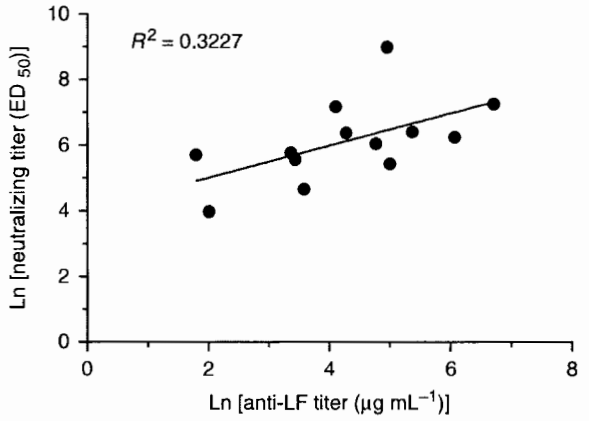

Fig. 3. Relationship of the anti-LF ELISA titer with the LT-neutralizing ED_{50} in cutaneous anthrax patients. There was a weak positive correlation ($R^2 = 0.3227$) between anti-LF IgG levels and neutralizing titer in infected individuals. No correlation was observed between anti-PA levels and neutralizing titer ($R^2 = 0.323$).

IgM/IgG toxin-specific antibody response at the first sampling point. In general, the IgM responses were considerably lower than the corresponding IgG levels for all patients, a pattern similar to that observed in AVA-immunized primates (Ivins *et al.*, 1998). Together, these data imply that class switching from IgM to IgG occurs early in the anthrax infection.

We observed differences in the ability of infected individuals to recognize toxin components. While 11 (65%) patients produced an LF-specific IgG response, only four (24%) mounted an IgG response to EF while only three (18%) demonstrated an IgG-specific immune response to PA. In those patients who showed the presence of anti-LF and anti-PA IgG antibodies (patients 2, 6 and 7), the LF titers exceeded the PA response by a factor of a least 3. Because both proteins are produced early in infection (Guidi-Rontani *et al.*, 1999), this disparity in response may reflect fundamental differences in how each protein is processed by the immune system. For example, LF is known to elicit higher IgG antibody titers than PA when administered to experimental animals (Price *et al.*, 2001; Hermanson *et al.*, 2004; Flick-Smith *et al.*, 2005).

Indeed, the overall disparity between anti-PA and anti-LF IgG responses observed in this study is likely to be the result of a combination of factors such as the virulence of the infecting strain, the route of infection and the health and genetic background of the individual. The initiation of antimicrobial therapy is also likely to have affected the level of toxin expression and may explain the absence of detectable response to any anthrax toxin component in six infected individuals (Stepanov *et al.*, 1996; Athamna *et al.*, 2004).

In an anthrax outbreak, it is crucial to identify infected individuals as quickly as possible to enable the initiation of timely treatment. The central role of the toxins in the progression of the infection has led to the proposal to use the antibody response to PA as a diagnostic marker of exposure to anthrax (Quinn *et al.*, 2002, 2004). This recommendation was based on the analysis of the PA-specific antibody response of patients with bioterrorism-related infections (Quinn *et al.*, 2004). While individuals with inhalation anthrax demonstrated an IgG response to PA as early as 11 days following the onset of symptoms, those with the cutaneous infections did not develop anti-PA titers until 21–34 days after the onset of symptoms and those titers did not peak until days 30–60 (Quinn *et al.*, 2004), confirming our findings that the anti-PA immune response develops slowly. Unlike our study, Quinn and colleagues did not screen their samples for the presence of an anti-LF immune response. If they had, they may have also found that the LF-specific antibody response preceded that of the PA response. We found that the LF-specific IgG was detected as early as day 4 after the onset of symptoms, and that all patients had developed anti-LF titers by day 15. In contrast, PA-specific IgG was first detected 13 days after the onset of symptoms, and had appeared in only three patients by day 21. The early production and prevalence of anti-LF antibodies after the development of symptoms suggest that the overall kinetics of the immune response is a rapid anti-LF response, followed by a slower, longer anti-PA response. The speed, strength and prevalence of the anti-LF response in infected patients clearly demonstrate that the presence of an anti-LF response might be a better diagnostic marker of infection than an anti-PA response.

We also sought to compare the response following immunization with the US AVA and UK AVP licensed anthrax vaccines. While both are subunit vaccines, they

differ primarily in their means of production. The current US licensed vaccine, AVA, is produced from a cell-free culture filtrate of an anaerobically grown *B. anthracis* strain V770-NP1-R (a nonencapsulated, nonproteolytic variant of a bovine strain isolated in Florida in 1951) and consists largely of PA adsorbed onto aluminum hydroxide (Baillie, 2001). In contrast, the UK vaccine, AVP, is produced from an alum precipitate of the cell-free culture filtrate of a static, aerobic culture of the *B. anthracis* Sterne strain 34F2. In addition to large amounts of PA, the vaccine also contains trace amounts of LF and other bacterially derived, immunogenic antigens, which have been shown to stimulate antibody responses in recipients and may contribute to protection (Baillie *et al.*, 2003, 2004; Whiting *et al.*, 2004). Indeed, the presence of these additional proteins may also account for the transient reactogenicity seen in some individuals (Turnbull, 2000). The efficacy of both vaccines has been demonstrated in numerous animal models including primates and has revealed a significant correlation between PA IgG titer and toxin-neutralizing activity, which is thought to be indicative of protection (Ivins *et al.*, 1998; Reuveny *et al.*, 2001; Little *et al.*, 2004; Phipps *et al.*, 2004; Quinn *et al.*, 2004; Pittman *et al.*, 2005).

In this study, we observed that the LF-specific human antibody was able to neutralize toxin activity. Several patients with high anti-LF IgG titers, but lacking anti-PA IgG titers had measurable toxin neutralization titers. This neutralization activity was not due to nonspecific protection as serum from six naïve individuals failed to protect macrophages (data not shown). Neither could neutralization be attributed to the presence of anti-PA antibodies, as samples obtained from patients 5, 8, 9, 10 and 17 showed no detectable anti-PA IgM or IgG antibodies, but still had quantifiable neutralizing activity. To our knowledge, this is the first report of the production of LF-specific neutralizing antibodies by humans following infection. The levels of neutralizing antibody generated by these individuals were roughly equivalent to the levels generated by AVP-immunized individuals (Hepburn *et al.*, 2007). The ability of LF to stimulate toxin-neutralizing antibodies is not surprising, given that LF must first bind to PA before it can be transported into the cytosol of the susceptible cell, where it exerts its toxic effects. Indeed, animal studies have demonstrated the ability of toxin-neutralizing LF-specific antibodies to protect rabbits against aerosol spore challenge (Zhao *et al.*, 2003; Hermanson *et al.*, 2004; Lim *et al.*, 2005). While we found no such correlation among our patients for PA, we did observe a weak correlation between LF-specific IgG and toxin-neutralizing titers. Because little is known about either the human response to anthrax or the ability of LF to generate protective antibodies, further work is required to determine the factors mediating LF-specific toxin neutralization.

Concerns over the ability to circumvent the current licensed vaccines by altering the antigenic structure of PA have spurred researchers to focus on additional vaccine targets (Schneerson *et al.*, 2003; Hoffmaster *et al.*, 2004). The immunogenicity of LF and its ability to stimulate the production of toxin-neutralizing antibodies makes a biologically inactive form of LF a strong candidate for inclusion in any future anthrax vaccine. We conclude from this preliminary study that an antibody response to LF might be a more sensitive diagnostic marker of anthrax than one to PA. In addition, the ability of human LF-specific antibodies to neutralize toxin activity supports the possible inclusion of LF in future anthrax vaccines.

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Authors'contribution

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